Abstract

**Purpose:** The objectives of this study were to investigate the effects of anti-vascular endothelial growth factor (VEGF) treatment on various vascular functions, gene expression, and growth of orthotopic human pancreatic cancer xenografts and thus to provide useful preclinical data for novel cancer treatments.

**Experimental Design:** Small pieces of a human pancreatic carcinoma, PANC-1, were implanted into the pancreas of male severe combined immunodeficient mice. The animals were treated with anti-human VEGF antibody A.4.6.1 (300 μg, every 3 days i.p.) or a nonspecific IgG between 4 and 8 weeks after tumor implantation. Then, vascular density, diameter, permeability, and tumor growth were determined by intravital microscopy. Subsequently, tumors were harvested, and angiogenic gene expression profile was determined by a microarray kit including 96 genes involved in tumor angiogenesis.

**Results:** Anti-VEGF antibody significantly reduced angiogenesis and growth of orthotopic PANC-1 tumors. In the anti-VEGF treatment group, the vessel density was significantly smaller (67.8 ± 10.6 cm/cm²) than that seen in the control group (10.6 cm/cm²). However, vessel diameter and permeability were not altered significantly by anti-VEGF antibody treatment. The pancreatic tumors in the treated group were significantly smaller than those in the control group. Microarray and subsequent Northern blot and semiquantitative reverse transcription-PCR analyses revealed both a decrease (fibroblast growth factor 1, transforming growth factor β1, platelet-derived growth factor α, erbB2, and c-ets1,) and an increase (placenta growth factor, hypoxia-inducible factor α, and endoglin) in expression of angiogenesis-related genes in the PANC-1 tumors by anti-VEGF treatment.

**Conclusions:** Anti-VEGF antibody treatment has differential effects on vessel functions as well as angiogenic gene expression and inhibitory effects on angiogenesis and growth of the orthotopic pancreatic tumor. Anti-VEGF strategy appears promising for pancreatic cancer treatment.

Introduction

Exocrine pancreatic cancer is now the fifth leading cause of cancer death in the United States, Japan, and Europe (1). Recent advances in the multimodality management of pancreatic cancer have lowered the mortality rates and improved the survival after surgery. However, the overall 1-year survival rate after diagnosis of pancreatic cancer remains below 20%, and the overall 5-year survival rate is only 3%, with the majority of patients dying of metastatic cancer recurrence (2). The growth and metastasis of solid tumors are angiogenesis dependent (3). VEGF is one of the most potent angiogenic factors (4). This multifunctional heparin-binding cytokine not only promotes angiogenesis by inducing proliferation, migration, and survival of endothelial cells but also increases vascular permeability and up-regulates leukocyte adhesion molecules on endothelial cells (4–6). In pancreatic cancer, VEGF is overexpressed and associated with disease progression (7). Based on successful preclinical efficacy, anti-VEGF antibodies and small molecule inhibitors directed toward the VEGFR have been introduced into clinical trials for selected tumors. However, most preclinical studies were carried out with ectopically (typically s.c.) growing tumors. These may not represent the true biology and treatment response of orthotopic tumors, due to differences in microenvironment (8, 9). We have recently established an orthotopic human pancreatic cancer xenograft model and found that the pancreas microenvironment promotes VEGF expression and tumor growth (10). The aims of this study were to determine the effects of anti-VEGF antibody treatment on angiogenesis, vascular permeability, and growth of orthotopic PANC-1 pancreatic tumors by intravital microscopy as well as angiogenic gene
expression profile by means of a microarray to provide useful information for understanding and designing pancreatic cancer treatment.

Materials and Methods

Cells, Animals, and Orthotopic Pancreatic Cancer Model. PANC-1 cells were derived from a poorly differentiated pancreatic ductal adenocarcinoma and obtained from the American Type Culture Collection (Manassas, VA). Cells were grown as adherent cultures in DMEM with 10% FBS in a humidified 5% CO2 atmosphere.

All animal procedures were fully approved by the institutional animal care and use committee. Male severe combined immunodeficient mice (25–30 g) were used. Before the procedures, mice were anesthetized with ketamine (90 mg/kg body weight) and xylazine (9 mg/kg body weight). The entire procedure was performed under sterile conditions inside a gnotobiotic animal facility in our department.

For the preparation of tumor source, PANC-1 cells were trypsinized to prepare single-cell suspensions, and a centrifuged pellet containing 1 × 10⁷ cells was then injected s.c. into severe combined immunodeficient mice. Tumors were allowed to grow for 6–8 weeks and then dissected. Small pieces of viable tumor tissue (approximately 1 mm in diameter) were used as a tumor source.

The orthotopic pancreatic cancer model was prepared as described previously (10). Briefly, the hair on the left flank was shaved, and a small left lateral laparotomy was performed. The splenic lobe of the pancreas was gently exteriorized from the abdominal cavity. A small piece of PANC-1 tumor was sutured to the serosal side of the pancreas with a 5-0 prolene (Ethicon, Somerville, NJ). The abdominal wall and the skin were sutured and closed with metal wound clips, respectively. Metal clips were removed 7 days after tumor implantation.

Measurements of Tumor Growth. Mice bearing orthotopic tumors were anesthetized, and laparotomy was performed. The size of the tumor was measured by a caliper. Tumor volume was calculated as π/6 × a × b × c (where a is longitudinal, b is short diameter, and c is thickness). These measurements were made at 4 and 8 weeks after tumor implantation for every animal.

Intravital Microscopy. Animals bearing tumors were anesthetized and observed by intravital microscopy 8 weeks after tumor implantation, ie, after 4 weeks of antibody treatment. Angiogenesis and vascular permeability were quantified by intravital microscopy as described previously (11, 12). Briefly, to visualize the blood vessels, 100 μl of a 10 mg/ml FITC-labeled dextran solution (M, 2,000,000; Sigma, St. Louis, MO) were injected i.v. Fluorescence images of five random locations of each tumor were recorded and digitized for subsequent off-line analysis. Functional vascular density was measured as the total length of perfused vessels per unit area of observation field (NIH Image version 1.6). NIH Image was also used for the measurement of the vessel diameter. The microvascular permeability to BSA was measured using tetramethylrhodamine-labeled BSA (0.1%, 100 μl; Molecular Probes, Eugene, OR) as described previously (12).

In Vivo Anti-VEGF Neutralizing Antibody Treatment. Four weeks after tumor implantation, orthotropic PANC-1 tumor-bearing mice were given murine antihuman VEGF neutralizing monoclonal antibody A.4.6.1 (a generous gift from Genentech, South San Francisco, CA) 300 μg/300 μl/mouse/i.p. every 3 days. As a control, the same amount of nonspecific murine IgG antibody was used. Treatment was continued for 4 weeks followed by intravital microscopy and sacrifice of the mice. Sixteen tumor-bearing animals were randomly assigned to each treatment group (control IgG, n = 9; anti-VEGF antibody, n = 7).

RNA Isolation. Total RNA was isolated from three different, randomly selected tumors of each group using Trizol (Life Technologies, Inc., Rockville, MD) according to the manufacturer’s specifications. The quality and integrity of RNA were checked by spectrophotometry and 1% ethidium bromide-agarose gel electrophoresis (Seakem GTG; FMC Bioproducts, Rockland, ME).

cDNA Array. For analysis of the differential expression of multiple genes, we used GEArray pathway-specific expression arrays (SuperArray, Inc., Bethesda, MD). For every tumor, 5 μg of collected total RNA were used in the cDNA probe synthesis with [32P]dCTP [6000 μCi; New England Nuclear Life Science Products, Inc. (Boston, MA)]. Purification, hybridization, and washings were done according to the manufacturer’s instructions (SuperArray, Inc.). Each GEArray membrane consisted of 96 coordinates containing specific cDNA fragments of genes involved in human angiogenesis, spotted in quadruplets as well as the negative pUC18 DNA, and β-actin and GAPDH for loading control. The spots were quantified using the free shareware Scanalyzer as well as GEArray software (SuperArray, Inc.). The relative abundance of a particular transcript was estimated by comparing its signal intensity with the signal derived from GAPDH after subtraction of pUC18 DNA intensity.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted by the single-step acid guanidinium thiocyanate phenol chloroform method. RNA was size-fractionated on 1.2% agarose/1.8 μm formaldehyde gels, electrotransferred onto nylon membranes, and cross-linked by UV irradiation. Primers for the cDNA probes of endoglin and PlGF were selected from GenBank and were as follows: (a) endoglin, ACC-TCT-GCT-CTG-AGC-TGA-AC (forward) and GTA-CTG-TGT-AGA-ACT-TGC-GGA-GGA-GGA (reverse); and (b) PlGF, AAG-CAG-AGA-CCC-ACA-GAC-T (forward) and GCA-GGA-TCC-GCA-TCC-CTA-CTT (reverse). cDNA probes were labeled with the Rediprime II Random Prime Labeling System (Amersham Biosciences United Kingdom Limited). Blots were prehybridized and hybridized with a cDNA probe of endoglin and PlGF. The blots were washed under high stringency conditions as reported by the manufacturer (Amersham Biosciences United Kingdom Limited). Blots were then exposed at 80°C to XAR-5 films (Eastman Kodak, Rochester, NY), and the resulting autoradiographs were scanned to quantify the intensity of the radiographic bands using the Fluorchem Visible Light Imaging (Q Imaging Corp., San Leandro, CA).

RT-PCR Analysis. RT-PCR was performed according to the procedure described by the manufacturer. Total RNA was isolated in the same manner as described above. RT-PCR was
performed using a RNA PCR kit from Invitrogen (THERMO-SCRIPT). The primers for amplification were synthesized on the basis of the coding region placed on the genechip. Samples for HIF-1α, c-ets, FGF1, TGF-β, and β-actin were denatured at 93°C for 3 min and then subjected to 35 cycles of 93°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final 10-min extension at 72°C in a Gene Amp PCR System 9600 (Perkin-Elmer/Cetus, Norwalk, CT), whereas samples for Erb-2 and PDGF were subjected to 30 cycles at 60°C. PCR products (10 μl) were analyzed on a 2% agarose gel in TAE buffer (40 mM Tris, 40 mM sodium acetate, and 1 mM EDTA (pH 8.4)) using a DNA ladder 100-bp marker. Primers were as follows: (a) HIF-1α, 5'-GGA-TGA-TGA-CTT-CCA-GTT-ACG-T-3' (forward) and 5'-TTC-CTC-AGG-AAC-TGT-AGT-TC-3' (reverse); (b) c-ets 1, 5'-ACC-TCG-GAT-TAC-TTC-ATT-AG-3' (forward) and 5'-TTC-TGC-AAG-GTG-TCT-GTC-TG-3' (reverse); (c) FGF1, 5'-AGC-CGG-TCC-TCG-GAC-TCA-CT-3' (forward) and 5'-AGA-CTG-GCC-AGC-CAG-TTG-ACT-3' (reverse); (d) TGF-β1, 5'-ACA-ATT-CCT-GGC-GAT-ACC-TC-3' (forward) and 5'-AGC-TGA-AGC-AAT-AGT-TGG-T-3' (reverse); (e) Erb-2, 5'-ACC-ATT-GAT-GTC-TAC-ATG-AT-3' (forward) and 5'-GAT-CTC-ACC-CAT-CAG-TAA-GAA-CA-3' (reverse); (f) PDGF, 5'-TAC-GAG-ATT-CCT-GCG-ACT-CAG-3' (forward) and 5'-ATC-CTC-ACC-TCA-CAT-CCG-TG-3' (reverse); and (g) β-actin, 5'-AGC-GCG-GCT-ACA-GCT-TCA-3' (forward) and 5'-TCT-CCT-TAA-TGT-CAA-GCA-CGA-3' (reverse).

Results

Anti-VEGF Neutralizing Antibody Decreased Vascular Density but not Diameter. Mice treated with the anti-VEGF neutralizing antibody showed significantly reduced angiogenesis. Vessel density was 67.8 ± 10.6 cm/cm² in the VEGF antibody-treated group and 146.7 ± 10.0 cm/cm² in the group treated with nonspecific IgG (P < 0.01; Figs. 1 and 2). However, VEGF antibody treatment did not alter vessel diameter (19.5 ± 2.0 and 19.6 ± 3.1 μm in treated and control tumors, respectively). As a result, vascular volume per unit area calculated from length and diameter of vessels tended to be smaller in the VEGF antibody-treated group compared with the control group (3.6 ± 0.7 and 6.8 ± 1.4 μm³/μm², respectively; P = 0.055; Fig. 2).

Anti-VEGF Neutralizing Antibody Did Not Decrease Vascular Permeability. Although we measured a permeability of 6.93 ± 2.4 × 10⁻⁷ cm/s in anti-VEGF neutralizing antibody-treated mice, the permeability was 11.4 ± 8.2 × 10⁻⁷ cm/s in the control group. However, there was no statistically significant difference between the two groups (P = 0.48; Fig. 3).
Anti-VEGF Neutralizing Antibody Decreased Tumor Growth. The VEGF antibody treatment reduced tumor growth. The pancreatic carcinomas in the treated group were statistically significantly smaller (P < 0.01; Fig. 4). In one of seven mice treated with the VEGF antibody, tumor regression was also observed.

Alteration in Angiogenic Gene Expression by Anti-VEGF Neutralizing Antibody. To detect possible gene expression profile alteration by anti-VEGF treatment, we investigated 96 genes involved in tumor angiogenesis by means of a microarray. Eight genes were differently expressed in the orthotopic PANC-1 tumors. The expression levels were normalized with housekeeping gene GAPDH and β-actin to compare different treatments. Erb-2, c-ets1, FGF1, PDGF, and TGF-β1 were down-regulated in the anti-VEGF neutralizing antibody-treated group (Fig. 5a). On the other hand, endoglin, HIF-1α, and PGF were up-regulated in the anti-VEGF neutralizing antibody-treated group. These data were validated by Northern blot analysis for PGF and endoglin (Fig. 5b) as well as semiquantitative RT-PCR for Erb-2, c-ets1, FGF1, PDGF, TGF-β1, and HIF-1α (Fig. 5c).

Discussion

Increased vascularity allows both an increase in tumor growth and a greater chance for hematogenous metastases (3). Fujimoto et al. (13) showed that the overexpression of VEGF correlated with high microvascular density and frequency of advanced stage of pancreatic cancer. We showed here that anti-VEGF neutralizing antibody treatment reduced vascular density in the orthotopic PANC-1 tumors compared with that in the control tumors treated with nonspecific IgG. Furthermore, we showed here a significant growth delay in the orthotopic pancreatic tumors with anti-VEGF antibody treatment parallel to the decrease in vascular density. These are in agreement with previous studies by ourselves and others in various tumor models (4, 6, 10, 14, 15). However, there was no significant difference in vessel diameter between the treated group and the control group. Furthermore, we were unable to show a statistically significant difference in vessel permeability between the mice treated with anti-VEGF neutralizing antibody and the control group. Both vessel diameter and vascular permeability remained high compared with those in normal pancreas (10). Anti-human VEGF antibody used in this study does not neu-

Fig. 3 Effect of anti-VEGF antibody treatment on vascular permeability. Bars, SE (anti-VEGF group, n = 7; control IgG group, n = 6).

Fig. 4 Effect of anti-VEGF antibody treatment on tumor growth. Four weeks after tumor implantation, the size of orthotopic pancreas tumors was measured by laparotomy (before treatment). Mice were then randomly assigned to anti-VEGF (n = 7) or control IgG (n = 9) treatment groups. Treatment was continued for 4 weeks, followed by tumor size measurement with laparotomy (after treatment). Bars, SE, * P < 0.05 as compared with control group by t test.

Fig. 5 Effect of anti-VEGF antibody treatment on angiogenic gene expression. a, microarray analysis of angiogenic genes. Genes altered more than 2-fold by anti-VEGF treatment are presented. Relative expression levels indicate positive or negative fold differences in anti-VEGF antibody-treated group compared with control antibody-treated group. b, Northern blot analysis of endoglin and PGF. IgG AB, control IgG treatment. Anti-VEGF AB, anti-VEGF antibody treatment. Left panels, endoglin and PGF were overexpressed 2- and 3-fold, respectively, in anti-VEGF-treated tumors compared with the control tumors. Right panel, the total RNA loading volume was shown on a 1% ethidium bromide gel. c, semiquantitative RT-PCR analysis of HIF-1α, c-ets1, FGF1, TGF-β1, Erb-2, and PDGF. Lanes M, DNA ladder, Lanes 1A–7A, anti-VEGF-treated samples; Lanes 1B–7B, IgG-treated samples. Lane 1, HIF-1α; Lane 2, c-ets1; Lane 3, FGF1; Lane 4, TGF-β1; Lane 5, Erb-2; Lane 6, PDGF; Lane 7, β-actin.
ralize VEGF from host stromal cells (mouse VEGF). Host-
derived VEGF or PlGF might compensate for loss of VEGF
signaling from tumor cells to some extent (16, 17). In fact, we
found that at a certain level of VEGF, the permeability in tumors
reaches an organ-dependent plateau that cannot be altered by
further increase in VEGF (17, 18). Also, there might be a
complex interplay between several growth factors, in which
VEGF plays an important role in physiological function, but alternate growth factors may control physiology once VEGF is
suppressed.

We have shown previously that VEGF has a direct effect
on PANC-1 cell proliferation (10). Because PANC-1 cells ex-
press receptors to VEGF (19, 20), VEGF may up-regulate
various angiogenic growth factors via autocrine/paracrine mech-
anisms. In fact, we found that anti-VEGF antibody treatment
reduced expression of multiple angiogenic factors and related
genes such as FGF1, PDGF, TGF-β1, Erb-2 and c-ets1. These
findings suggest better treatment outcome with anti-VEGF treat-
ment if the tumor cells express functional VEGFRs. However,
on the other hand, some angiogenic growth factors may be
up-regulated as a consequence of treatment stress (21). In this
study, we found up-regulation of endoglin, HIF-1α, and PlGF
by anti-VEGF antibody treatment. Reduced vessel density by
anti-VEGF antibody treatment may result in increased hypoxia
in the treated tumors. Hypoxia induces HIF-1α expression as
well as its protein stabilization, which then mediates a series of
transcriptional responses to adapt to hypoxic condition includ-
ing VEGF up-regulation. Although we did not see a significant
change in human VEGF (tumor derived) expression level by the
treatment, presumably due to a combination of microenviron-
mental regulation (hypoxia) and direct effect of the treatment of
tumor cells, hypoxia can induce VEGF expression in host stro-
mal cells (murine VEGF) and thus may mask the effects of the
treatment on vessel growth (diameter) and permeability.

Endoglin (CD105) is a cell membrane glycoprotein, a
receptor for TGF-β1, and overexpressed on highly proliferating
endothelial cells in culture and on endothelial cells of angio-
genic blood vessels within benign and malignant tissues. In
addition to endothelial cells, it is also expressed on tumor cells,
mesangial cells, smooth muscle cells, monocytes, and macro-
phages to a certain extent. Increased expression of endoglin in
tumor cells after anti-VEGF treatment may potentiate TGF-β1-
induced reduction of tumor cell proliferation. However, the
impact of endoglin alteration in our model is not clear because
tGF-β1, the ligand of endoglin, decreased simultaneously.

Increased expression of PlGF after anti-VEGF treatment is
a particularly interesting finding in this study and was also
reported in a rhabdomyosarcoma xenograft model (21). It is
believed that PlGF stimulates VEGF signaling by displacing
VEGF to “functional VEGFR2” from the “VEGFR1 sink” (22).
Recently, however, there is a growing body of evidence that
VEGFR1 actually acts as functional receptor rather than sink,
especially under pathological conditions (such as in tumors),
and that VEGFR1-specific ligand PlGF induces vascular per-
meability and angiogenesis (22). Analysis of S’ region of murine
and human PlGF gene as well as in vitro functional analysis
revealed that hypoxia induces PlGF expression via metal tran-
scription factor 1, and this induction is potentiated by Ras
oncogene, which is expressed in most pancreatic cancers, in-
cluding PANC-1 (23). Increased PlGF may explain, at least in
part, the discrepancy in the effects of anti-VEGF treatment on
vessel density, diameter, and permeability in our model. Differential functions of VEGFRs such as branching angiogenesis,
luminal growth, and vascular hyperpermeability are still ill
defined. Additional studies on the interaction between PlGF and
VEGF as well as the resulting vascular phenotype are warranted
(24).

We have shown here that anti-VEGF neutralizing antibody
treatment in orthotopically grown pancreatic carcinoma induces
differential effects on various vascular functions as well as the
angiogenic gene transcriptional profile and inhibits angiogenesis
and tumor growth. We may expect an even more profound effect
in a clinical setting because antihuman VEGF antibody blocks
both tumor and host-derived VEGF (25). We conclude that
anti-VEGF treatment has a significant effect on the progression
of disease in orthotopic pancreatic cancer. Our findings strongly
support anti-VEGF strategies in the treatment of patients with
pancreatic cancer. Our data also suggest the importance of gene
expression profiling during cancer treatment. With advances in
the detection system and the availability of various treatment
reagents, gene profiling during treatment should allow tailored
treatment in near future.

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